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The Orally Active Antihyperglycemic Drug β-Guanidinopropionic Acid Is Transported by the Human Proton-Coupled Amino Acid Transporter hPAT1

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Abstract: The orally administered creatine analogue β-guanidinopropionic acid (β-GPA) decreases plasma glucose levels by increasing the sensitivity to insulin. This effect is based on a β-GPA induced expression of mRNA and total protein content of the insulin-responsive glucose transporter GLUT4. Although the oral availability of β-GPA is well established, the underlying uptake mechanism has not yet been studied. We investigated whether the H^+-coupled amino acid transporter PAT1, which is expressed in the apical membrane of intestinal cells, accepts guanidine derivatives as substrates. Uptake of L-[^{3}H]proline into Caco-2 cells expressing hPAT1 constitutively was strongly inhibited by β-GPA and its derivatives guanidinoacetic acid (GAA) and 4-guanidinobutyric acid (4-GBA). Competition assays revealed apparent affinity constants of about 1.5 mM. Electrophysiological measurements at hPAT1-expressing *Xenopus laevis* oocytes unequivocally demonstrated that β-GPA, GAA and 4-GBA are effectively transported by this transport system in an electrogenic manner. We conclude that hPAT1 might be responsible for the intestinal absorption of β-GPA thereby allowing its oral administration. Moreover, with β-GPA we identified a new high affinity hPAT1 substrate that might be an interesting starting point for future drug design-drug delivery strategies.

Keywords: Antihyperglycemic; β-guanidinopropionic acid (β-GPA); human H^+-coupled amino acid transporter hPAT1; Caco-2 cells; *Xenopus laevis* oocytes; drug delivery; intestinal barrier; voltage clamp

Introduction

The orally administered creatine analogue β-guanidinopropionic acid (β-GPA) is used to decrease plasma glucose levels by improving the sensitivity to insulin.[1,2] The effect is based on a β-GPA induced decrease in the intramuscular content of ATP, phosphocreatine and total creatine[3,4] which leads to increased expression of insulin-responsive glucose transporter GLUT4 mRNA and total protein content in rat and mouse skeletal muscle tissue,[5,6] an effect that has also been seen in trained humans.[7] β-GPA reduces hyperglycemic levels comparable to or even more potent than the biguanidine metformin which is extensively used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM).[1,8] Other guanidine derivatives such as guanidinoacetic acid (GAA) and diaminoguanidinoacetic acids have also been shown to be effective in lowering plasma glucose levels.[1,2]

Even though the oral availability of β-GPA is well-known, the underlying uptake mechanism has not yet been studied.

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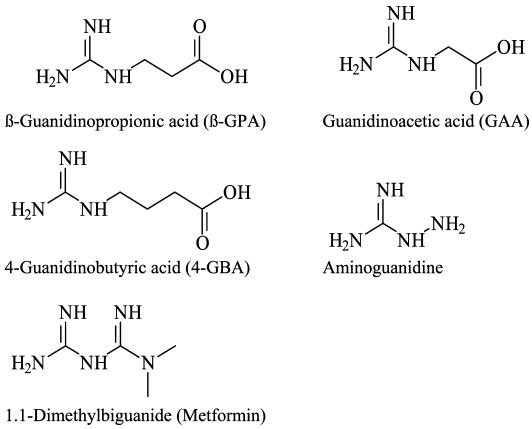

Figure 1. Chemical structures of investigated guanidine derivatives.

Judging from its molecular structure (Figure 1) we hypothesized that β-GPA and related guanidine derivatives might be substrates for the H^+-coupled amino acid transporter PAT1. This membrane transporter has been characterized on a molecular level in 2002 and was assigned to family 36 of the solute carriers (SLC36A1).[9] Its cDNA was isolated first from rat brain[10] and subsequently from mouse intestine[9] and human Caco-2 cells.[11] PAT1 is expressed in the apical membrane of intestinal epithelial cells, but hPAT1 mRNA was also detected in brain, colon, liver and lung.[11,12] The system acts H^+-dependent, Na^+-independent and rheogenic. Compared to the isoform transporter PAT2, it can be classified as a "low affinity, high capacity" transporter.

PAT1 transports a variety of substrates preferring small unbranched amino and imino acids such as L-proline and L-alanine as well as several derivatives (e.g., γ-aminobutyric acid and taurine). Very recently, PAT1 gained much interest because of its ability to transport drugs such as inhibitors of collagen biosynthesis (e.g., 3,4-dehydro-D,L-proline, L-azetidine-2-carboxylic acid), the anticraving agent 3-amino-1-propanesulfonic acid, D-cycloserine and GABA derivatives such as vigabatrin thereby allowing their oral administration.[13−15]

In the present investigation we studied the interaction of β-GPA and the guanidine derivatives GAA and 4-guanidinobutyric acid (4-GBA) with hPAT1 at Caco-2 cells expressing this transporter constitutively. Their transmembrane transport was studied electrophysiologically in hPAT1-expressing *Xenopus laevis* oocytes.

Materials and Methods

Materials. β-GPA and substances and chemicals for buffer solutions were purchased from Sigma-Aldrich (Taufkirchen, Germany). The intestinal cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Media and supplements were from PAA (Cölbe, Germany), and the fetal bovine serum was from Biochrom (Berlin, Germany). L-[^{3}H]Proline (specific radioactivity 100 Ci/mmol) was supplied by ARC (American Radiolabeled Chemicals, Inc., St. Louis, MO).

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The *X. laevis* oocyte expression vector pNKS was kindly provided by G. Schmalzing (RWTH, Aachen, Germany).

Cell Culture. Caco-2 cells were cultured (passages 62−93) in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution and gentamicin (50 μg/mL) as described earlier.[15] Cells at 80−90% density were released using trypsin/EDTA (0.05%/0.02%) and subcultured in 35 mm disposable Petri dishes. With a starting cell density of 0.8×10^6 cells per dish, the cultures reached confluence within 24 h. Uptake experiments were performed on the seventh day after seeding.

L-[^{3}H]Proline Uptake Measurements. L-[^{3}H]Proline uptake was measured as described previously,[15] using uptake buffer (1 mL) containing 25 mM MES/Tris (pH 6.0) or 25 mM HEPES/Tris (pH 7.5) with 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 10 nM L-[^{3}H]proline and unlabeled compounds. After a 10 min incubation time, cells were quickly washed four times with ice-cold buffer, solubilized and prepared for liquid scintillation spectrometry. Protein was measured according to the procedure of Bradford.

Construction of pNKS-hPAT1. The pNKS-transducin vector contains the 5′ and 3′ UTRs of the *X. laevis* oocyte β-globin gene and the cDNA of murine transducin. The latter was cut out of the vector using the restriction enzymes *Aat*II and *Xba*I. To clone the transporter's cDNA into pNKS, *Aat*II and *Xba*I restriction sites were introduced at the 5′ and 3′ ends by PCR, respectively. As template, the wildtype cDNA of human PAT1 in the pSPORT1 vector was used.[16] After restriction enzyme digestion the PCR product was ligated into the digested pNKS vector. The insertion of the correct cDNA was verified by sequencing.

In Vitro cRNA Synthesis. The pNKS-hPAT1 construct served as templates for cRNA synthesis. After linearizing the plasmid with *Not*I cRNA was synthesized using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX). The cRNA was purified with the aid of the MEGAclear kit (Ambion), and the concentration was determined by UV absorbance at 260 nm.

***X. laevis* Oocytes Expressing hPAT1 and Electrophysiology.** Oocytes were surgically removed from anesthetized *X. laevis* frogs, dissected and defolliculated as described by Riedel and co-workers.[17] The removed oocytes were were manually selected and injected with 25 ng of cRNA. Water injected oocytes were used as control. All electrophysiological measurements were performed after incubating oocytes for 5−6 days at 19 °C in modified Barth's medium (5 mM HEPES/NaOH pH 7.4, 100 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10000 U/mL penicillin and 10 mg/mL streptomycin). To record two-electrode voltage clamp signals oocytes were placed superfused with oocyte Ringer (ORi) buffer (10 mM MES/Tris pH 6.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM KCl) in the absence or presence of substances at a concentration of 20 mM. Quick and reproducible solution exchanges were achieved using a small tubelike chamber (0.1 mL) combined with fast superfusion (75 μL/s).[18,19] Microelectrodes with resistances between 0.8 and 1.4 MΩ were made of borosilicate glass and filled with 3 M KCl. Whole-cell currents were recorded and filtered at 100 Hz using a two-electrode voltage-clamp amplifier (OC-725C, Hamden, USA) and sampled at 85 Hz. Oocytes were voltage clamped at a membrane potential of −60 mV. Current−voltage ($I-V$) relations were analyzed by voltage ramps going from −120 mV to + 60 mV within 240 ms separated by 580 ms pauses with a clamp potential of −60 mV. $I-V$ measurements were carried out in the presence of 20 mM β-GPA at varying pH values (5.5 to 7.5 in ORi buffer: 10 mM MES/Tris or HEPES/Tris, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM KCl). The Michaelis constants (K_t) were derived from experiments using ORi buffer containing increasing concentrations of glycine (0−100 mM) and β-GPA (0−50 mM). Currents generated by substrate transport at a given membrane potential were calculated as the difference of the currents measured in the presence and absence of substrates.

Data Analysis. Results are given as means ± SEM. Nonlinear regression analysis, calculation of inhibition constants (K_i) from IC_{50} values, computation of Michaelis constants and statistical analysis (ANOVA followed by Bonferroni) were done as described.[15] Mean values obtained from oocyte experiments were calculated from measurements of 4−8 (as stated in the legend to Table 1) oocytes from two batches, and the kinetic constants were obtained by nonlinear regression of the Michaelis−Menten plot (SigmaPlot). Oocyte currents were analyzed using the Superpatch 2000 program (Julius Bernstein Institute of Physiology, SP-Analyzer by T. Böhm, Halle, Germany).

Results

Interaction of Pharmaceutically Relevant Guanidine Derivatives with hPAT1 in Caco-2 Cells. To investigate the interaction of guanidine derivatives with hPAT1 we first determined the inhibition of hPAT1 mediated L-[^{3}H]proline uptake in Caco-2 cells by β-GPA, GAA, 4-GBA, aminoguanidine and 1.1-dimethylbiguanide (metformin) in excess amounts (10 mM). As shown in Figure 2A, total uptake of L-[^{3}H]proline (606.8 ± 20.7 fmol/10 min per mg protein)

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Table 1. Parameters of Transport of Guanidine Derivatives by hPAT1[a]

	Caco-2 cells		*X. laevis* oocytes
substrate/inhibitor	% L-[^{3}H]proline uptake	K_i (mM)	% I_{Gly}
glycine	56 ± 2[b]	5.0 ± 0.6[b]	100 ± 15
L-proline	32 ± 4[b]	1.7 ± 0.1[c]	122 ± 18
β-GPA	14 ± 1	1.3 ± 0.1	140 ± 19
GAA	16 ± 1	1.5 ± 0.1	117 ± 16
4-GBA	15 ± 2	1.4 ± 0.1	138 ± 16
aminoguanidine	88 ± 3	>30	39 ± 5
metformin	90 ± 4	>30	8.3 ± 1.6
L-tryptophan	48 ± 4[b]	4.7 ± 0.3[b]	1.7 ± 0.4

[a] Uptake of L-[^{3}H]proline (10 nM) in Caco-2 cells was measured at pH 6.0 for 10 min in the absence (100%) or presence of unlabeled compounds (10 mM). K_i values were calculated from curves shown in Figure 2B. Percent I_{Gly} in oocytes was calculated from the recordings of the $I-V$ relationships representing the current evoked by 20 mM of the compounds divided by that generated by 20 mM glycine (I_{Gly} = 161 ± 24 nA) at hPAT1 expressing *X. laevis* oocytes at a membrane potential of − 60 mV. Percentage of glycine current was calculated by relation of the second glycine-evoked current to the first one. (*n*: 4−8.) [b] Values from ref 20. [c] K_t value.

measured at an outside pH of 6.0 was strongly inhibited by β-GPA, GAA and 4-GBA by 86, 84 and 85%, respectively. Only a very slight inhibition was observed using aminoguanidine and metformin.

To determine apparent affinity constants (K_i), uptake of L-[^{3}H]proline was measured at increasing concentrations (0.1−31.6 mM) of β-GPA, GAA and 4-GBA (Figure 2B, Table 1). β-GPA, GAA and 4-GBA displayed affinity constants of 1.3, 1.5, and 1.4 mM, respectively, and can therefore be classified as high affinity inhibitors. As expected, L-[^{3}H]proline uptake at pH 7.5 is just about 1/7 of the uptake at pH 6.0 and K_i for β-GPA interaction is here barely measurable (K_i > 30 mM).

Nonlinear regression of the data shown in Figure 2B (pH 6.0) revealed Hill coefficients of ∼1 indicating a competitive type of inhibition. To study the effect of β-GPA on the kinetic parameters of L-proline uptake in detail, L-proline uptake as a function of extracellular L-proline concentration was investigated in the absence and presence of β-GPA at a concentration close to its K_i value (1.5 mM). In the absence of β-GPA, the Michaelis constant (K_t) of L-proline uptake was 1.7 ± 0.1 mM and the maximal velocity of transport (V_{max}) was 185.4 ± 7.8 nmol/10 min per mg protein. In the presence of 1.5 mM β-GPA the K_t value increased by a factor 2 (3.2 ± 0.2 mM) whereas V_{max} was not affected (176.8 ± 9.7 nmol/10 min per mg protein; Figure 3).

Two-Electrode Voltage-Clamp Experiments Using hPAT1 Expressing *X. laevis* Oocytes. Competition assays using radiolabeled reference substrates give important information about interaction of a compound with an uptake process. They do not allow conclusions about actual transport of the competing compounds. To investigate hPAT1 mediated translocation, the two-electrode voltage-clamp technique in *X. laevis* oocytes was applied. Figure 4A shows the currents obtained after application of compounds (all 20 mM)

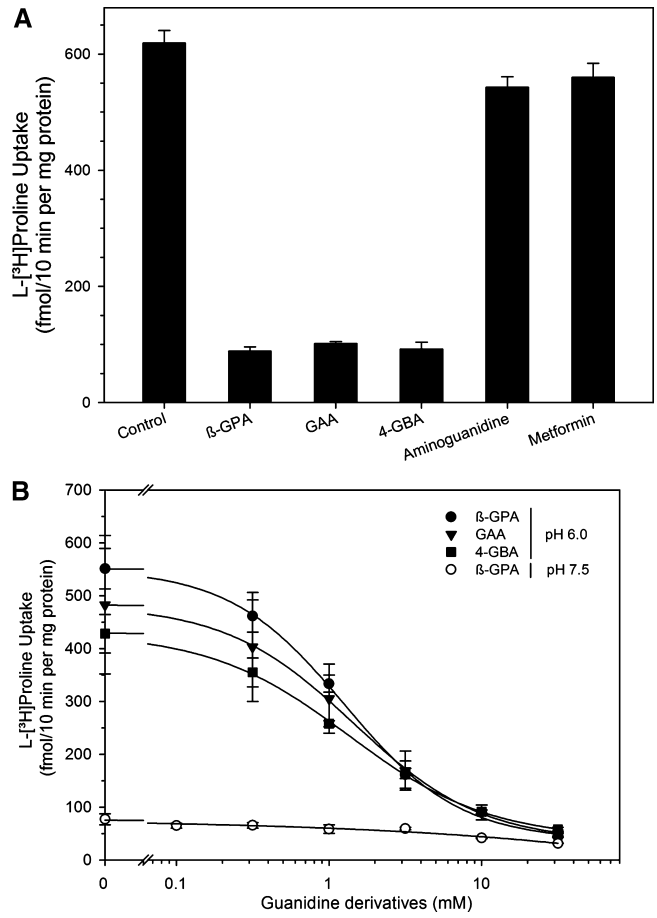

Figure 2. Inhibition of L-[^{3}H]proline uptake in Caco-2 cells. (A) Uptake of L-[^{3}H]proline (10 nM; 10 min; pH 6.0) was measured in the absence and presence of guanidine derivatives at a fixed concentration of 10 mM (pH readjusted if necessary). n = 4. (B) L-[^{3}H]Proline uptake (10 nM; 10 min) was measured in uptake buffer containing increasing concentrations of unlabeled β-GPA, GAA and 4-GBA. In the absence of inhibitors, L-[^{3}H]proline uptake was 487.4 ± 35.6 fmol/10 min per mg protein at pH 6.0 and 77.0 ± 10.4 fmol/10 min per mg protein at pH 7.5. n = 4−6.

to oocytes at an extracellular pH of 6.5 and a membrane potential of −60 mV. The prototype PAT1 substrates glycine and L-proline induced high inward currents (Table 1). Importantly, β-GPA, GAA and 4-GBA elicited comparable currents. Aminoguanidine induced a smaller but significant and metformin a tiny current (Figure 4A, Table 1). Similarly, L-tryptophan, a potent nontransported hPAT1 inhibitor,[20] elicited no currents. In water-injected oocytes, used as controls, glycine, L-proline and the guanidine derivatives did not induce any currents (Figure 4A). To demonstrate the dependency of hPAT1-mediated β-GPA transport on an inward directed H^+-gradient, current−voltage ($I-V$) relations at pH 5.5, 6.5, and 7.5 were recorded (Figure 4B). The highest inward directed currents were elicited at pH 5.5. Michaelis−Menten kinetics for β-GPA and glycine performed at a membrane voltage of −60 mV at pH 6.5 revealed K_t values of 5.6 ± 0.5 mM and 10.9 ± 0.7 mM, respectively.

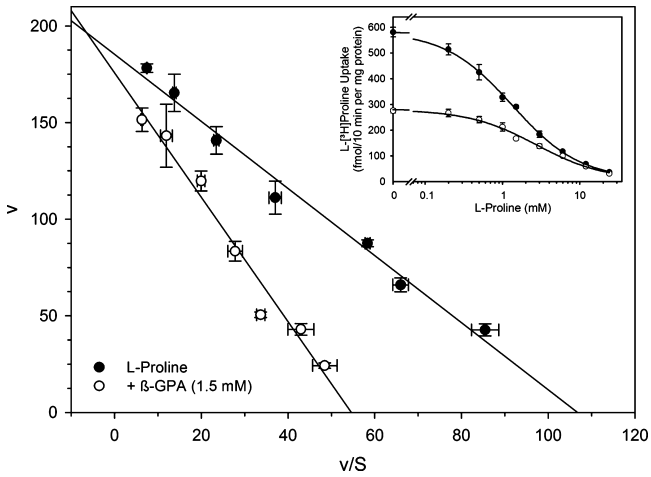

Figure 3. Effect of β-GPA on the substrate saturation kinetics of L-proline uptake in Caco-2 cells. Uptake of L-proline was measured for 10 min at pH 6.0. Eadie−Hofstee transformation of the data was performed after the correction for the linear transport component (determined experimentally; v = uptake rate in nmol/10 min per mg protein; S = L-proline concentration in mM). Inset: Untransformated data of L-[^{3}H]proline uptake inhibition in the absence and presence of β-GPA (1.5 mM) by increasing concentrations of L-proline. $n = 4$.

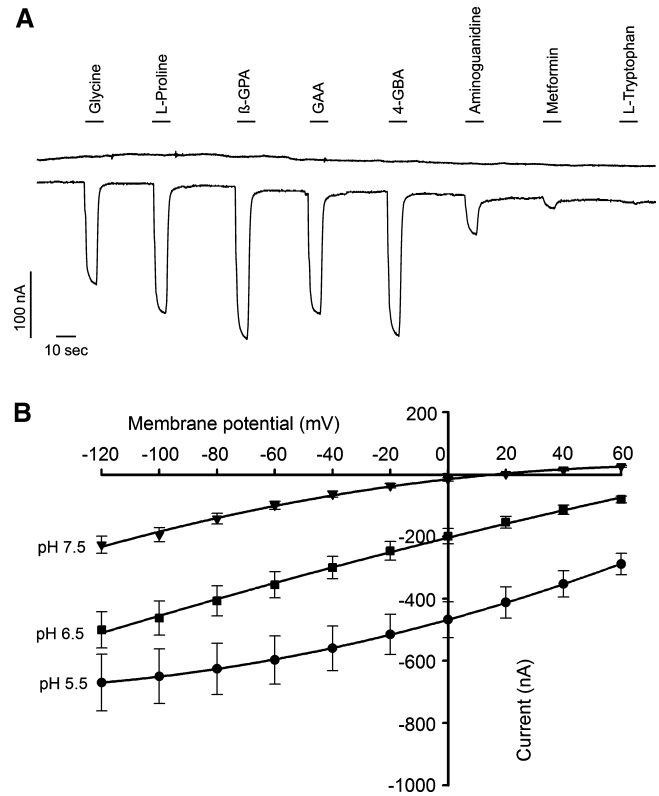

Figure 4. Electrophysiological analysis of hPAT1 function in *X. laevis* oocytes. (A) Lower trace: Currents induced by amino acids and guanidine derivatives (20 mM) in oocytes expressing hPAT1. Upper trace: measurement in a water-injected oocyte. (B) pH dependence of β-GPA induced currents. $I-V$ relationships were measured by voltage ramps in the presence of β-GPA (20 mM) using ORi buffer with varying pH values (5.5, 6.5, and 7.5). n = 5−6.

These data clearly show that β-GPA, GAA and 4-GBA are not only recognized by hPAT1 but also translocated across the cell membrane in an electrogenic mode.

Discussion

β-GPA is an endogenous metabolite found in animals and humans.[21] The use of guanidine derivatives in the treatment of diabetic indications such as hyperglycemia was first described ninety years ago.[22] The transmembrane transport of such drugs across the brush-border membrane of the human small intestinal epithelium is the most critical step during absorption. Both its broad substrate specificity and its localization in the apical membrane of enterocytes make the H^+-coupled amino acid transporter PAT1 a promising candidate for oral drug delivery. The focus of the present study was to investigate the interaction of orally administered antihyperglycemic guanidine derivatives with hPAT1.

Our data on the inhibition of proline uptake by the antihyperglycemic guanidine derivatives β-GPA, GAA and 4-GBA, the structural template compound aminoguanidine and the widely used metformin showed that β-GPA, GAA and 4-GBA interact with hPAT1 with high affinity. Aminoguanidine and metformin are not recognized by the system. The affinity constants in the lowest millimolar range correspond very well to the concentration that can be expected in the fluid compartment facing the membrane protein: The recommended oral dose of β-GPA is 5−100 mg/kg/day.[23] Assuming an oral dose of 3 g of β-GPA per day (0.35−7 g), a luminal concentration of around 3 mM is very conceivable.

To determine whether β-GPA is indeed transported by hPAT1 we used the two-electrode voltage-clamp technique in *X. laevis* oocytes. It demonstrated that the guanidine derivatives β-GPA, GAA and 4-GBA evoked currents similar to those induced by prototype PAT1-substrates. This transport of β-GPA by hPAT1 at therapeutic concentrations makes it very likely that PAT1 functions as an important if not the major β-GPA transporter but does not rule out the involvement of other transport systems. In the human intestine, many different transport systems with diverse transport modes and functionalities but sometimes overlapping substrate specifici-

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ties are expressed. Since β-GPA is structurally related to creatine, it could, for example, also be a substrate for the creatine transporter CRT (SLC6A8).[24] With regard to transport mechanism and substrate specificity, CRT and PAT1 are two very different systems, but they are both expressed in the intestine at apical cell membranes and they both recognize β-GPA although with very different affinity constants. CRT transports its substrates in a Na^+- and Cl^+-dependent mode.[24] Peral and co-workers[25] characterized the intestinal CRT as a high affinity transporter with a K_t value for creatine of 29 μM. They also showed that CRT interacts with β-GPA ($K_i \approx 25$ μM) but not with glycine, taurine or β-alanine. Actual transport of β-GPA by CRT has not been shown. Even if β-GPA is transported, CRT might be completely saturated under therapeutic β-GPA concentrations. More importantly, the V_{max} values for transport of prototype substrates differ significantly by a factor of about 330, PAT1 being the high capacity transport system.[25] Therefore we believe that PAT1 is, at least at the intestinal epithelium, the major β-GPA transporting system.

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Conclusions

The orally active antihyperglycemic agent β-GPA is recognized and transported by the H^+-coupled amino acid transporter PAT1. The intestinal hPAT1 is very likely responsible for β-GPA absorption thereby allowing its oral administration. Moreover, with β-GPA, GAA and 4-GBA we identified a new high affinity hPAT1 substrate group that might be an interesting starting point for future drug design−drug delivery strategies.

Abbreviations Used

β-Guanidinopropionic acid, β-GPA; guanidinoacetic acid, GAA; 4-guanidinobutyric acid, 4-GBA; non-insulin-dependent diabetes mellitus, NIDDM; H^+-coupled amino acid transporter 1, PAT1.

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